

EXHIBIT J

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Genetic and biochemical characterization of dTOR, the *Drosophila* homolog of the target of rapamycin

Sean Oldham^{1,3}, Jacques Montagne^{2,3},
Thomas Radimerski², George Thomas²,
and Ernst Hafen^{1,4}

¹Zoologisches Institut, Universität Zürich, 8057 Zürich, Switzerland; ²Friedrich Miescher Institute, 4058 Basel, Switzerland

The adaptation of growth in response to nutritional changes is essential for the proper development of all organisms. Here we describe the identification of the *Drosophila* homolog of the target of rapamycin (TOR), a candidate effector for nutritional sensing. Genetic and biochemical analyses indicate that dTOR impinges on the insulin signaling pathway by autonomously affecting growth through modulating the activity of dS6K. However, in contrast to other components in the insulin signaling pathway, partial loss of dTOR function preferentially reduces growth of the endoreplicating tissues. These results are consistent with dTOR residing on a parallel amino acid sensing pathway.

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During the development of unicellular and multicellular organisms, growth is dependent on the integration of diverse intracellular signals, which are triggered by patterning and/or environmental cues (Oldham et al. 2000). The availability of nutrients strongly influences growth of single cells and multicellular organisms and in some cases specifies alternative developmental programs. For example, in yeast, nitrogen or carbon deprivation leads to withdrawal from vegetative growth, arrest in G₀, and up-regulation of autophagy (Johnston et al. 1977; Ohsumi 1999). Likewise, in the nematode *Caenorhabditis elegans*, limiting nutrients or overcrowding elicits an alternative developmental program termed the dauer stage (Riddle 1997). In this stage, the animal remains sexually immature and stockpiles additional lipids for survival during unfavorable growth periods (Kimura et al. 1997). In more complex organisms such as insects and mammals, an alternative developmental program is lacking. Instead, nutrient limitation is managed by delaying development and, in severe cases, reducing the final body

size of the organism. In the case of *Drosophila*, when nutrients become limiting, available resources are mobilized toward maintaining the growth of the mitotic tissues, which during metamorphosis form smaller, yet still fertile, flies (Robertson 1963).

In metazoans, one component of the complex physiological response of energy homeostasis and growth control is the insulin and IGF signaling system (Efstratiadis 1998; Patti and Kahn 1998). Insulin and IGF activate two main signaling pathways via the insulin receptor substrates (IRS1–4): the Ras/MAPK pathway, which is involved in proliferation, and the phosphatidylinositol 3-kinase (PI3K) signaling pathway, which is involved in cell growth, survival, and metabolic homeostasis (White 1998; Rameh and Cantley 1999). PI3K mediates its effects on downstream signaling components through the production of phosphatidylinositol 3,4,5-tris phosphate (PIP₃), which acts to recruit pleckstrin homology (PH) domain-containing proteins like protein kinase B (PKB) (Chan et al. 1999; Meier and Hemmings 1999). The actions of activated PI3K are antagonized by the 3'-phospho-inositol specific lipid phosphatase encoded by the tumor suppressor gene *PTEN* (Maehama and Dixon 1999). An important protein implicated in the PI3K signaling pathway is the target of rapamycin (TOR). TOR has been reported to be regulated by PKB (Scott et al. 1998). Initially, TOR1 and TOR2 were identified in yeast as mutations that conferred resistance to the antiproliferative effects of rapamycin (Heitman et al. 1991). Rapamycin is an antibiotic that inhibits both yeast TOR and mammalian TOR (mTOR, also known as FRAP or RAFT) function by forming an inhibitory complex with the immunophilin, FK506 binding protein-12 (FKBP12), that binds to a region adjacent to the kinase domain termed the FKBP12-rapamycin binding domain (FRB; Brown et al. 1994; Sabatini et al. 1994; Thomas and Hall 1997; Cutler et al. 1999). TOR is most related to the ATM/DNA-PK family of checkpoint protein kinases and is more distantly related to the PI3K family (Thomas and Hall 1997; Cutler et al. 1999).

A key downstream target of mTOR function is protein synthesis. In part, mTOR positively mediates protein synthesis by modulating the activities of important translational components, including the translation initiation factor 4E binding proteins (4E-BP1-3) and the ribosomal protein S6 kinases (Chou and Blenis 1995; Lawrence and Abraham 1997; Dennis et al. 1999). Under conditions of reduced nutrients, such as amino acid limitation, mTOR negatively regulates protein synthesis and positively up-regulates autophagy (Dennis et al. 1999). Thus, mTOR may serve as a nutritional checkpoint for cell growth and ultimately, proliferation.

Little is known concerning the role of TOR during metazoan development. Here we (see also, Zhang et al. 2000, this issue) describe the genetic and biochemical characterization of the *Drosophila* homolog of TOR (dTOR). We find that the strong dTOR mutants have similar characteristics to upstream components in the

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³These authors contributed equally to this work.

⁴Corresponding author.

E-MAIL: hafen@zool.unizh.ch; FAX 41-1-635-68-20.

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dPI3K branch of the insulin pathway. Weak *dTOR* mutants, however, display a unique phenotype. Unlike larvae mutant for components of the insulin signaling pathway, imaginal disks of larvae with reduced *dTOR* function grow to their normal size at the apparent expense of the endoreplicating tissue.

Results and Discussion

We recently established a tissue-specific genetic screen for recessive mutations affecting cell growth and proliferation in the *Drosophila* compound eye. In this screen, genetically mosaic flies are generated in which the eye and head capsule are homozygous for a randomly induced mutation, while the rest of the body and the germ line are heterozygous and, thus, phenotypically wild type. Remarkably, mosaic flies containing a mutation in a growth-promoting gene have eye and head structures that are strongly reduced in size relative to their wild-type sized heterozygous bodies and are termed pinhead flies (Fig. 1A). Two EMS-induced pinhead mutations (*2L1* and *2L19*; Fig. 1B,C) mapped to chromosomal position 34A, where the *Drosophila* homolog of TOR (*dTOR*) is located (Adams et al. 2000). These mutations fail to complement two lethal P-element insertions, *EP(2)2353* and *l(2)k17004* (Török et al. 1993; Rørth et al. 1998; Spradling et al. 1999), located 262 and 211 bp, respectively, upstream of the putative translation start site of *dTOR* (Fig. 1E). The *dTOR* genomic region spans ~10 kb and is composed of seven exons (7.4 kb cDNA) encoding a 2480-amino acid protein with a predicted M_w of 282 kD (Fig. 1E). The *dTOR* protein exhibits 35%, 38%, 35%, and 41% overall amino acid identity to yeast TOR1, TOR2, *C. elegans* TOR, and *Arabidopsis thaliana* TOR, respectively. The overall identity with mTOR is significantly higher (56%) and is especially conserved in the kinase and FRB domains (74% and 77%). Sequence analysis of DNA from flies heterozygous for the EMS-induced *dTOR* mutations revealed two nucleotide substitutions. The lesion of *dTOR*^{2L1} results in a change of a proline to a leucine at amino acid position 2303 (P2303L; Fig. 1E). The location of this mutation within a highly conserved region of the kinase domain implies that the kinase activity of *dTOR* is critical for its function, as has been shown for the yeast TORs (Zheng et al. 1995). In contrast, the lesion in *dTOR*^{2L19} is an arginine changed to a nonsense mutation at amino acid residue 248 (R248Stop), giving rise to a stop codon. This mutation would be predicted to result in a short, truncated protein and should thus be a complete loss-of-function mutation.

The phenotypes associated with the complete loss of *dTOR* function are remarkably similar to phenotypes associated with mutations in the insulin receptor (*Inr*) pathway. First, strong *dTOR* mutants arrest development at a similar stage as do strong mutants in the *Inr* pathway or amino acid-starved larvae with little detectable imaginal tissue (see below). Second, *dTOR* mutant clones have a significant proliferative disadvantage similar to *Inr* pathway mutant clones (data not shown).

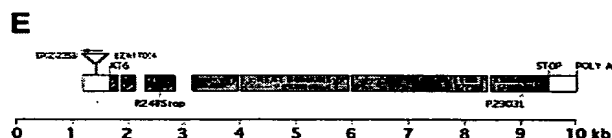
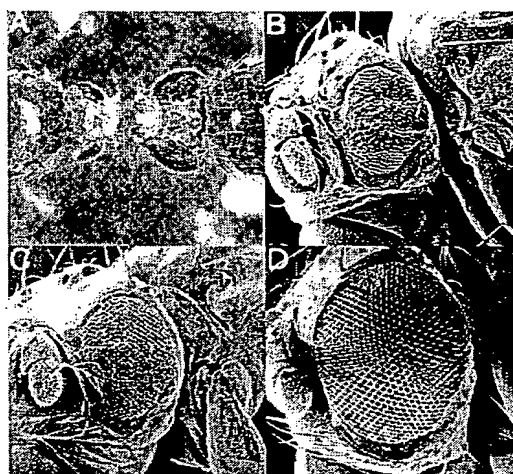


Figure 1. Identification of *dTOR*. Tissue-specific induction of *dTOR*^{2L1} and *dTOR*^{2L19} mutant clones using the ey-Flp technique (Newsome et al. 2000) produced flies with reduced head size relative to their body. (A) Dorsal view of *y w* ey-Flp; P(w+) l(2)2L-3.1 FRT40/CyO *y*⁺ (right) and *y w* ey-Flp; *dTOR*^{2L1} FRT40/P(w+) l(2)2L-3.1 FRT40 (left). Flies of the following genotype were examined by SEM: (B) *y w* ey-Flp; *dTOR*^{2L19} FRT40/P(w+) l(2)2L-3.1 FRT40; (C) *y w* ey-Flp; *dTOR*^{2L1} FRT40/P(w+) l(2)2L-3.1 FRT40; (D) wild-type eye. Bar, 100 μ m. (E) Genomic structure and mutants of *dTOR*. The grey region consists of the coding region, and the gaps indicate introns. The percentage amino acid identity of each domain to human TOR is shown in parentheses.

Clones of *dTOR* null mutant cells, although severely affected, are not cell lethal. Similarly, in most mammalian cell types, rapamycin decreases but does not abolish cell growth, except for IL2-mediated T-cell proliferation (Chou and Blenis 1995). Third, the strict autonomous control of cell growth without disturbing the specification and differentiation is also seen with the *dTOR* mutants. Indeed, loss of *dTOR* function in clones of homozygous mutant cells in the adult eye showed that only the mutant cells, as exemplified by the dark, circular rhabdomeres, are severely reduced in size (Fig. 2C). Analysis of imaginal wing disk cells at the end of the third larval instar by fluorescence-activated cell sorting (FACS), confirmed that cells from the weak heteroallelic combination, *dTOR*^{2L1}/*dTOR*^{l(2)k17004}, are smaller than those of wild type (Fig. 2B). The effect on cell size is more pronounced in G₁ than G₂ (data not shown), consistent with mTOR function having a predominate role on cell growth during G₁ (Terada et al. 1993; Barbet et al. 1996). Despite this observation, there is no apparent difference between the distribution of *dTOR* mutant and wild-type cells within each phase of the cell cycle (Fig.

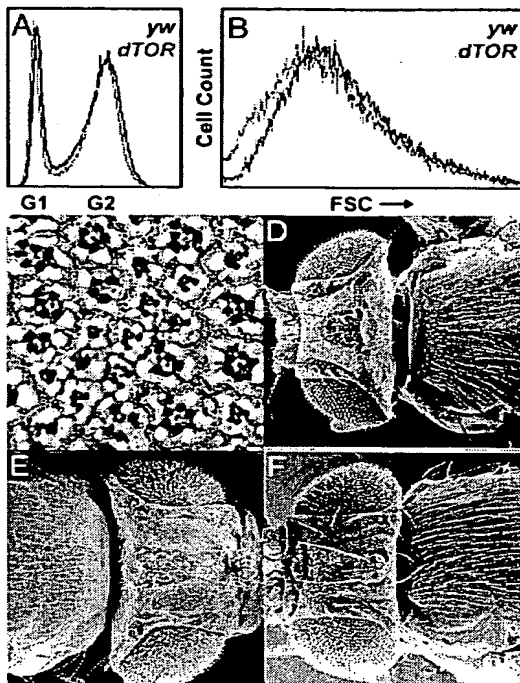


Figure 2. *dTOR* acts autonomously in the control of cell growth in the eye. (A,B) FACS analysis of *dTOR*^{2L1}/*dTOR*^{1(2)k17004} mutant wing disks showing forward scatter (FSC) and cell cycle distribution. (C) Section through an eye containing a mitotic clone of *dTOR*^{1(2)k17004}. The area of the mutant clone is demarcated by the absence of red pigment. At the boundaries of the clone, ommatidia consist of mixed wild-type heterozygous and homozygous mutant cells. (D–F) Flies were examined by SEM. (D) *yw* control. (E) Loss of *dPTEN* function in the eye and head capsule results in a fly with a big head. (F) The large head caused by loss of *dPTEN* function in the eye can be suppressed by homozygosity for *dTOR*^{EP(2)2353}. Bar, 200 μ m.

2A). Although the similarities of the loss-of-function mutant phenotypes of *dTOR* and other components of the *Inr* pathway are consistent with a model in which *dTOR* acts downstream of *dPI3K*, the analysis of partial loss-of-function *dTOR* mutants and the biochemical analysis of *dS6K* activity (see below) indicates a more complex relationship between *dTOR* and the *Inr* pathway.

We examined genetic interactions between *dTOR* mutants and other mutations in the insulin pathway. *dPTEN* encodes a negative effector of insulin signaling (Goberdhan et al. 1999; Huang et al. 1999), and eyes and heads lacking *dPTEN* function are significantly larger than wild-type heads (Fig. 2D,E; Gao et al. 2000). Removal of *dTOR* function strongly reduced the size of *dPTEN* mutant heads (Fig. 2F), suggesting that *dTOR* is required for the increased growth generated by the loss of *dPTEN* function.

In vertebrates, *S6K* activity is blocked by rapamycin, an inhibitor of TOR. Therefore, we analyzed *dS6K* activity in immunoprecipitates of extracts from larvae mu-

tant for *dTOR*, *dS6K*, *chico*, and larvae treated with rapamycin or deprived of amino acids. A severe reduction in the phosphorylation of ribosomal protein *S6* was observed in extracts from strong *dTOR*^{2L1}/*dTOR*^{2L19} mutant larvae (Fig. 3, cf. lanes 1 and 3). This was not caused by a reduction in *dS6K* protein as shown by Western blotting of these extracts (Fig. 3). In addition, we consistently noted that the *dS6K* protein was up-regulated in the *dTOR* mutant larvae and amino acid-starved larvae. In all cases, Western blot analysis showed equivalent amounts of initiation factor 4E (eIF-4E; Fig. 3). *dS6K* activity was not detected in *dS6K*¹⁻¹ null mutants and was severely reduced when wild-type larvae were starved for amino acids or treated with rapamycin (Fig. 3, lanes 2,4,6). Higher doses of rapamycin blocked development during early larval stages, leading to lethality (data not shown). Analysis of the weak *dTOR*^{2L1}/*dTOR*^{1(2)k17004} or *dTOR*^{2L1}/*dTOR*^{EP(2)2353} heteroallelic combinations also revealed a reduction in *dS6K* activity in the third larval instar and an up-regulation of the protein as compared with wild-type flies (Fig. 3, cf. lane 7 with lanes 10 and 11). The surprising fact that *dTOR* mutants and amino acid starvation result in an up-regulation of *dS6K* levels suggests that *dTOR* and amino acids may negatively control the protein levels of *dS6K*. Unexpectedly, *dS6K* activity as well as protein levels were unaffected in *chico* mutants (Fig. 3, lane 9). It may be that *Inr* does not signal to *dS6K* or that *dS6K* resides on a parallel pathway that bifurcates upstream of *Chico*. In support of the latter possibility, *Inr* has been shown to genetically interact with *dPI3K* independently of *Chico* (Boehni et al. 1999), presumably through docking sites for the *p60* adaptor of *dPI3K* in the *Inr* C-terminal tail. This result suggests that there is a *dS6K* independent pathway for growth control and that the reduced *Inr*-mediated *dPI3K* signaling in a *chico* mutant is sufficient for *dS6K* activation.

The biochemical differences between the ability of *Chico* and *dTOR* to activate *dS6K* argue for a more complex relationship between the *Inr* pathway and *dTOR*. Given the low number of pharate adults, we compared the weight of *dTOR*, *dS6K*¹⁻¹, and *chico* mutants at an early pupal stage (Fig. 4A). The weight of the *dTOR* mutant pupae is more similar to *dS6K* than to *chico* mutant pupae (Fig. 4B). Thus, in the absence of *dS6K* function or the presence of reduced *dTOR* levels, cellular growth rates are diminished but larvae pupariate at a larger size

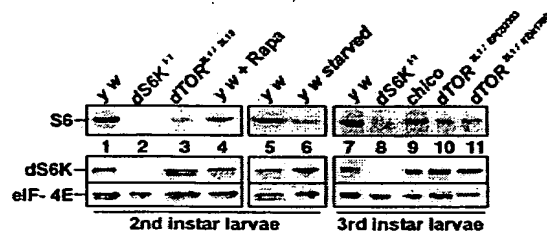


Figure 3. *dTOR* mutations and amino acid starvation affect the level and activity of *dS6K*. In vitro kinase assay to assess the levels of *dS6K* activity (top) and Western blot of the protein levels of *dS6K* and *Drosophila* eIF-4E (bottom).

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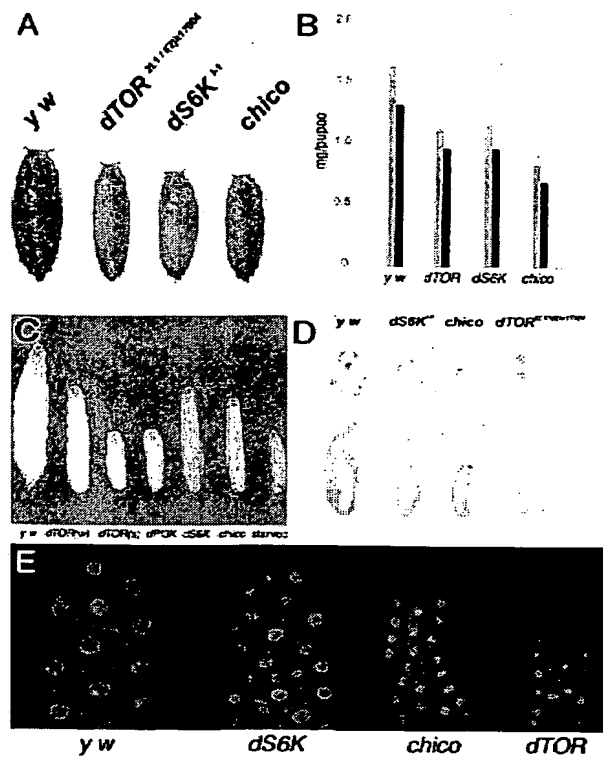


Figure 4. *dTOR* larval and pupal mutant phenotypes. (A) Pupal sizes of the various mutant combinations. Pupae were collected just after puparium formation. (B) Pupal weights of the various mutant combinations. Gray bars are female, black bars are male. (C) Heteroallelic combinations of *dTOR* mutant larvae compared with mutants of other components in the *Inr* pathway. All larvae were taken from a staged collection at day 5. *dTOR*(w) is *dTOR*^{2L1}/*dTOR*^{1(2)k17004}, *dTOR*(s) is *dTOR*^{2L1}/*dTOR*^{2L19}, *dPI3K* is a null mutation (Weinkove et al. 1999), and *dS6K* is *dS6K*¹⁻¹. (D) The size of mutant disks and salivary glands of hypomorphic *dTOR* mutants are distinct from *dS6K* and *chico*. (E) DAPI and phalloidin staining of salivary glands from *yw*, *dS6K*¹⁻¹, *chico*, and *dTOR*^{2L1}/*dTOR*^{1(2)k17004} mutant larvae. All disks and salivary glands were taken from larvae that had everted their spiracles just before pupation.

as a result of a longer developmental delay. Importantly, *dS6K* mutant flies have cells that are smaller but of the normal number (Montagne et al. 1999). However, in *chico* mutants, pupariation is initiated at a much smaller size. The result is that *chico* mutants emerge after only a 2-d delay and are smaller than *dTOR* and *dS6K* mutants because of fewer and smaller cells (Boehni et al. 1999; Montagne et al. 1999; data not shown). Therefore, while insulin signaling controls cell size and cell number, *dS6K* primarily controls cell size. It will be of interest to know whether *dTOR* is also limited to controlling only cell size.

Larvae are composed of mitotic cells, largely represented by the imaginal disks, and of endoreplicating tissues, which form larval structures like the gut, fat body, and salivary glands. An increase in DNA ploidy of larval

cells is required for the ~200-fold increase in mass obtained by the larvae during the 5-d period between the completion of embryogenesis and the beginning of pupation (Church and Robertson 1966). During starvation, larvae sacrifice their endoreplicating tissue to maintain the growth and proliferation of the mitotic cells that are required to form the reproductive adult (Britton and Edgar 1998). Furthermore, *dS6K* activity is reduced in starved larvae and *dTOR* mutants (Fig. 3). These observations prompted us to analyze the mitotic and endoreplicating tissues of *dTOR*, *dS6K*, and *chico* mutant larvae just before pupariation. Strong *dTOR* and *dPI3K* mutants, as well as amino acid-starved larvae, are incapable of growth and have barely detectable imaginal and endoreplicating tissues (Fig. 4C; Weinkove et al. 1999; data not shown). Surprisingly, the wing disks of the weak *dTOR* heteroallelic combination were of approximately equivalent size to that of wild-type larvae, whereas those of *dS6K*¹⁻¹ mutants were reduced (Fig. 4D). However, the amount of endoreplicating tissue in the *dTOR* mutant as compared to wild-type larvae was severely decreased. This is clearly demonstrated by comparing the salivary glands of *dTOR* mutant and wild-type larvae (Fig. 4D). In contrast, the size of endoreplicating tissue and imaginal disks in *dS6K* null mutants as well as *chico* null mutants was reduced in size to approximately the same extent (Fig. 4D). Staining of the salivary glands with DAPI and phalloidin revealed that the size of the nuclei and, thus, the degree of endoreplication was severely reduced in *dS6K*, *chico*, and *dTOR* mutants (Fig. 4E). The difference in size between *dTOR* and *dS6K* mutant salivary glands is largely caused by a very pronounced reduction in cytoplasmic volume in *dTOR* mutants. The nuclear to cytoplasmic ratio was higher in *dTOR* salivary glands than in *yw*, *dS6K*, or *chico* mutant salivary glands (4.5, 2.25, and 2.1 times, respectively). Thus, it appears that partial loss of *dTOR* function permits the growth of imaginal tissue to wild-type size, while endoreplicating tissue is disproportionately reduced, a phenotype distinct from *dS6K* mutants. Consistent with this finding, we were unable to rescue the lethality of the different *dTOR* mutants by constitutive expression of a *S6K1* variant, *D₃E-E₃₈₉*, which exhibits high basal activity in the absence of mitogens (Dennis et al. 1996) under the control of the α -tubulin promoter, which rescues all aspects of the *dS6K*¹⁻¹ null phenotype (Montagne et al. 1999; data not shown). Therefore, *dS6K*-independent processes must contribute to the weak *dTOR* phenotype.

The effect of rapamycin and amino acids on translation is mediated through the *S6Ks* and the 4E-BPs (Dennis et al. 1999). Unlike the other elements in the *PI3K* signaling pathway, absence of amino acids blocks both *S6K* activation and 4E-BP phosphorylation (Blommaert et al. 1995; Hara et al. 1998). Indeed, a mutant of *S6K1*, lacking a portion of both its amino and carboxyl termini, is resistant to rapamycin but still sensitive to the fungal metabolite wortmannin, an inhibitor of *PI3K*. This suggests that the *PI3K*-dependent signal to *S6K* activation does not involve *TOR*. This same mutant is also unaffected by amino acid withdrawal, consistent with the

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role of mTOR as an amino acid checkpoint in S6K activation (Hara et al. 1998; Dennis et al. 1999). Although there is some controversy concerning the ability of mitogens to activate mTOR, we find, as have others (Gingras et al. 1999, cited in Hara et al. 1998), that the in vitro activity of mTOR from cultured cells toward either itself, S6K1, or 4E-BP1 is unaffected by mitogens (P.B. Dennis and G. Thomas, unpubl.). Thus, mTOR may act as a permissive signal that primes 4E-BP phosphorylation and S6K activation by the PI3K signaling pathway if amino acids, and possibly other nutrients, are at sufficient levels (Hara et al. 1998; Gingras et al. 1999). Likewise, in *Drosophila* larvae, amino acids are necessary, but not sufficient, for imaginal disk and endoreplicating tissue proliferation (Britton and Edgar 1998), compatible with dTOR acting in a parallel pathway involved in amino acid sensing (Fig. 5). The fact that *chico* mutant larvae have normal levels of dS6K activity and that the dTOR larval phenotypes with respect to the imaginal disks and endoreplicating tissues are so distinct compared with other mutants in the Inr pathway, supports the possibility that dTOR is not responsive to insulin signaling.

It is well established in yeast that TOR is an important mediator of nutrient limitation, and it has been proposed that TOR acts as an amino acid effector to coordinate the response of yeast to different nutritional conditions (Barbet et al. 1996). Indeed, the similarities between dTOR mutant larvae and larvae deprived of amino acids are striking. Therefore, it is likely that dTOR also functions

as an amino acid sensor in multicellular organisms. The fact that yeast and *Arabidopsis* do not have an insulin system suggests that TOR may be an ancestral and widespread nutritional sensor. To provide additional levels of control, it may have been integrated into the insulin system later to respond to different modes of nutrient deprivation with different developmental responses.

Materials and methods

Molecular characterization of dTOR

We used the ey-FLP method (Newsome et al. 2000) for a tissue-specific F₁ screen for recessive mutations affecting cell growth and proliferation. Two EMS-induced alleles (*dTOR*^{2L1} and *dTOR*^{2L19}) that produce flies with small heads relative to their heterozygous, phenotypically wild-type sized bodies were identified and failed to complement two P-element insertions in the 5' region of *dTOR*. The genomic structure was predicted by GENSCAN and GADFLY (GENSCAN, <http://genes.mit.edu/GENSCAN.html>; BDGP, <http://www.fruitfly.org>).

Clonal and larval analysis

Clonal analysis was done as described (Boehni et al. 1999). The following genotypes were used to compare mosaic clones of *dPTEN* versus *dPTEN-dTOR* mutants: *y w ey-Flp; P(w+) l(2)2L-3.1 FRT40/FRT40 dPTEN*¹¹⁷ and *y w ey-Flp; P(w+) l(2)2L-3.1 FRT40/FRT40 dPTEN*¹¹⁷, *dTOR*^{EP(2)2353}. For imaginal disk/salivary gland and FACS analysis, late third instar larvae just before pupariation were examined as previously described (Montagne et al. 1999). Imaginal disks and salivary glands were dissected and fixed in paraformaldehyde as described (Montagne et al. 1999). DAPI and phalloidin staining were performed following standard procedure and analyzed by confocal imaging. The nuclear and cytoplasmic volumes were derived from nuclear radial and internuclei measurements, respectively, from 10 independent salivary glands. These measurements were converted to volumes using the formula $V = 4/3\pi r^3$. The cytoplasmic volume was determined by subtracting the nuclear volume from the total cell volume.

S6K assay

Amino acid-starved larvae were obtained as described (Britton and Edgar 1998). For the rapamycin (Calbiochem) feeding, 24 h after egg laying, 100 μ L of a 40 μ M rapamycin solution was added once a day for up to 6 d. Five days AED, rapamycin-fed larvae were developmentally delayed at the second instar. Larvae were extensively washed, frozen in liquid nitrogen, and squashed in extraction buffer (Stewart et al. 1996). Cell debris was removed by centrifugation and for each dS6K activity assay, 40 μ g of total protein extract was immunoprecipitated using 10 μ L of a monoclonal antibody generated by NOVARTIS, using a fusion protein between maltose binding protein (New England Biolabs) and the carboxy-terminal amino acids 435–490 of dS6K. After incubation with 20 μ L of protein-G Sepharose (Amersham Pharmacia), immuno complexes were sedimented, extensively washed, and assayed for [³²P] P_i incorporation as previously described (Stewart et al. 1996). Western blotting was performed with polyclonal antibodies directed against the amino terminus of dS6K (Stewart et al. 1996) or the *Drosophila* eIF4E (provided by J.M. Sierra, Centro de Biología Molecular Severo Ochoa).

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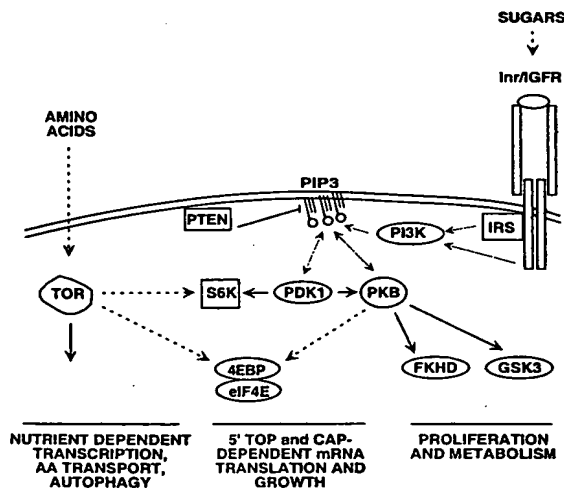


Figure 5. Model showing the possible relationship of dTOR with the insulin pathway. The unexpected finding that hypomorphic mutations of *dTOR*, but not null mutations of *chico*, strongly inhibit dS6K activity may be explained by a two-threshold model. The threshold for dS6K inactivation by dTOR is low, whereas that for dS6K inactivation by the Inr pathway is high. Thus, a low level of PI3K signaling from Inr may be sufficient for dS6K activation in the absence of Chico. Dashed arrow indicates indirect or unclear interaction. Solid arrow indicates direct interaction.

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